GASTRULAR BLOCKAGE IN FROGS' EGGS PRODUCED BY OXYGEN POISONING[1]

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Seldom do embryos stop developing without dying. Occasionally, however, maintenance becomes temporarily independent of gross morphological change as in the diapause of insects. Although non-reversible, this condition may be experimentally produced in amphibians by a few methods which result in highly uniform populations of arrested embryos which stay alive, that is, do not cytolyze for a relatively long time. Well-known among these techniques are CN^- or azide treatment (Spiegelman and Moog, 1945), and certain hybridizations (Moore, 1941; Brachet, 1944). In the late forties, however, Nelsen (1947, 1948, 1949, 1950) obtained a gastrular block in frogs' eggs by using 3 atmospheres of oxygen added to air at standard pressure. This method offers certain advantages toward a causal analysis of development.

First, the agent's effect is not immediate as in the case of azide, nor is it necessary to treat the embryos continuously as with CN^-. After 24 hours of treatment with oxygen pressure, the embryos are in the early cleavage stages. Until late blastulation they cannot be distinguished from the controls. Development stops just before dorsal lip formation and cytolysis does not set in for at least 30 hours. Embryos are thus obtainable with chemical aberrations which have not yet appeared at the morphological level.

Second, while each of the other methods except that employing azide has an all-or-none effect, the influence of oxygen pressure may be varied by controlling the dosage. By reducing the latter, "incompletely blocked" embryos are produced. These develop into larvae of normal appearance except for the scar of an abnormal gastrulation in the form of a persistent yolk plug.

Third, azide will affect any pre-gastrular stage. Although CN^- and hybridization have effects which are largely specific for gastrulation, oxygen pressure's specificity is even sharper. Clayton (1950) has shown in embryos pre-treated with the appropriate dose of oxygen that the movements of the notochord anlage are not prevented, though some of the other gastrular movements cease. Thus, incompletely blocked embryos do form neural tubes. With either CN^- or hybridization, however, gastrulation is completely blocked and all the movements stop.

Before exploiting these advantages afforded by the oxygen pressure effect, certain preliminary problems needed clarification. Accordingly, the present report is

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an investigation of 1) whether it is oxygen or pressure or both that affects the embryos, 2) dosage requirements, and 3) whether there is any effect on developmental rate prior to the gastrular block as in the case of CN^-. Although the latter does not stop pre-gastrular development, the fact that it slows these stages shows that it attacks a system on which development is dependent before as well as during gastrulation and thus detracts from its value as an analytical tool for the study of chemical events peculiar to the process of gastrulation.

Materials and Methods

Eggs of *Rana pipiens* from Vermont were obtained by the pituitary injection method of Rugh (1948, pp. 102–106) and fertilized by his method with slight modifications. After injection the females were kept at 12° to 14° C. until stripped; insemination was at room temperature. Fertilization and development of the embryos was in 10% frog Ringer's solution.

A few experiments were performed shortly after the natural breeding season of *R. pipiens*. For this purpose gravid females were stored at 5° C. from January until used as above. These "summer" frogs were kept in a 0.03% tap water solution of sodium sulfadiazine until injection.

Stages of development were designated according to Shumway (1940) and with reference to Rugh (1948, pp. 63–65). The designated stage was the latest one which at least one-half the embryos had reached.

Apparatus

An apparatus was constructed consisting of 6 glass pressure chambers which were shaken and kept at constant temperature. The chambers were continuous or discontinuous with each other in various combinations. Thus 6 levels of either of 2 variables could be studied at the same time, that is, on samples of a single clutch of eggs. These dosage variables were pressure and hours of treatment. In addition it was possible to have 3 chambers stationary with simultaneous shaking of the others. This permitted the effect of shaking to be determined on one clutch of embryos at 3 levels of either of the 2 dosage variables. Since the apparatus included only one water bath, comparative temperature studies on single clutches were precluded. Provision was made for the inclusion of 4 control bottles, shaken and unshaken, and containing eggs under no increased oxygen pressure.

The pressure manifold with its 3 gauges was mounted above a rectangular Warburg apparatus (Fig. 1). The latter provided the temperature regulation and shaking mechanism needed. Rubber pressure tubing connected the manifold to the pressure chamber assemblies submerged in the water bath. On each bank of the Warburg apparatus were mounted a few manometer supports connected by means of an aluminum rod fastened to their horizontal arms. Shaking of the pressure chambers was effected by clamping the assemblies to this rod.

The pressure chamber assemblies (Fig. 2) were slightly modified units of the Parr hydrogenation apparatus. In each assembly, a 500-ml. Pyrex glass bottle (surrounded by a perforated steel shield) served as the pressure chamber for the embryos.

The pressure manifold (Fig. 1) was assembled of $\frac{1}{4}$-inch galvanized iron pipe and brass fittings. The nozzles of the manifold and of the pressure chamber inlet

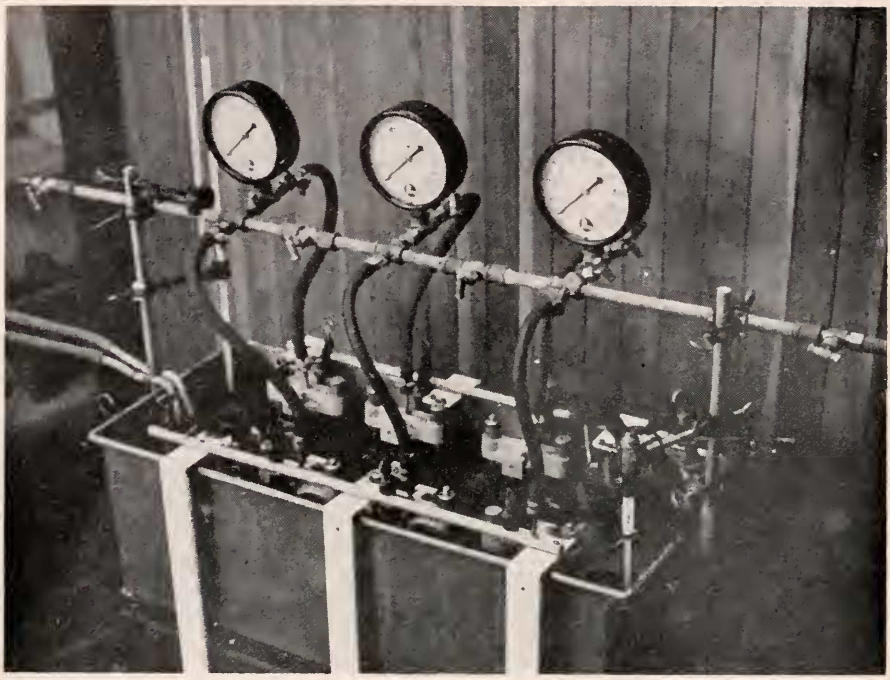

FIGURE 1. Constant-temperature pressure apparatus with provision for shaking.

tube permitted a flexible connection of rubber pressure tubing and this, in turn, allowed for shaking of the pressure chamber assemblies while the manifold was stationary. The gauges supplied by the Parr Instrument Co. were of the Bourdon type and read from 0 to 100 p.s.i. (pounds per square inch) in units of 1 which were large enough for estimation of the needle position to the nearest 0.2 p.s.i.

FIGURE 2. Pressure chamber assembly.

They had a specified accuracy of at least ± 0.5 p.s.i. When in a closed system, with gas supplied from a single source, the gauge readings up to 50 p.s.i. agreed to 0.2 p.s.i. The 10 valves with stainless steel needles were from Hoke, Inc. A commercial pressure cylinder supplied the gas which entered the manifold through either terminal valve; by shutting the other one, a closed system was effected. To decompress the first pressure chamber in a time series the appropriate terminal valve was opened. For the others in the series, non-terminal valves were used. The specified purity of the gases obtained from the Ohio Chemical and Surgical Equipment Co. and from the Matheson Co. was 99.5%. The principal impurity of the oxygen was nitrogen and vice versa. For each experimental run, the settings of the needle valves were determined by the variables to be studied.

The system permitted positive pressures up to 50 p.s.i. The total leakage during the course of any run never exceeded 5% of the gauge readings registered at the start. Actually, more than 2% leakage seldom occurred.

Temperature control was maintained as in ordinary procedures with the Warburg apparatus, with one exception. Since all the runs were below room temperature, cooling coils were added to the floor of the water bath. Through these coils flowed water which was cooled in a separate water bath by a portable refrigerator. This "cooling bath" was temperature-controlled at about 5° C. below the temperature desired in the Warburg bath. The heating unit of the latter operated intermittently against this continuous cooling. The Warburg bath was run at 18.0°, 12.0°, or 8.0° C. with a variability of ± 0.05° C. in each case.

Shaking was at a rate consistent with normal development of the embryos and rapid gas diffusion between the liquid phase containing the eggs and the gas phase above it. The stationary surface area was 4.9 square inches and 50 ml. of 10% Ringer's solution were used. The depth in the pressure bottle of the 10% Ringer's solution plus the embryos was 1 inch. The Warburg was altered to permit shaking on each bank at 30 ± ½ c.p.m. in a horizontal plane with amplitude of 1½ inches. In a few runs with a preliminary apparatus, the shaking rate was 36 ± 1 c.p.m.

Procedure

Each experimental run may be generally divided into 3 phases: 1) fertilization and compression, 2) decompression and selection of embryos, and 3) tabulation of abnormalities. Table I summarizes these steps.

1) At room temperature and 30–45 minutes after insemination, the clutch of eggs is rinsed with 10% Ringer's solution and then cut up into groups of 20–40 eggs. Only those clutches of eggs in which at least 80% rotate are used. The animals are distributed about 300 to a pressure bottle, each of the latter containing 50 ml. of 10% Ringer's solution. Including one control, 7 bottles are usually loaded. The metal-shielded bottles are fitted to the rubber stoppers attached to the manifold of the apparatus, then the clamps to the bottles. The former are tightened and, with the addition of the control bottle(s), are attached to the aluminum rods, thus submerging them all in the water bath, the latter at 18.0°, 12.0°, or 8.0° C. Now shaking is begun and pressure is built up gradually and simultaneously in all the bottles over a period of 20 minutes. The midpoint of this period is noted as the start of pressure treatment. The bottles are not flushed; the oxygen is added to the air in them; hence the total pressure in each bottle

equals the sum of the gauge reading for oxygen plus 1 atmosphere of air. After application of pressure the remaining eggs are observed to make sure that the first cleavage has not yet occurred. The bottles are shaken continuously until the time for decompression except in certain experiments where the effect of shaking is studied.

2) Decompression is gradual over one hour, and the midpoint of this period of time is taken as the end of pressure treatment. The order and times of decompression of the individual chambers in any one run varies, of course, with the purpose of the latter. While pressure is being released, work is progressively begun on the contents of each bottle already decompressed and immediately un-

TABLE I

Summary of procedure

Phases	Steps	Temperature changes of eggs	Relative time approximately$^{\Delta}$		Stage of development of normal eggs
			First	Last	
			Bottle		
1.	Bath on Fertilization Rotation Loading Shaker on Compression	 Room temp. Bath temp. 	 0 min. 45 min. 45 min.–100 min. 100 min.–120 min.		 Before 3
2.	Decompression Unloading Fresh Ringer's Selection (Fresh Ringer's)	 Room temp. 18° C.	11–12 hrs. 13 hrs.	16–17 hrs. *(A) *(B) 19 hrs.	 Cleavage 8, 9
3.	Tabulation	Room temp.	71–74 hrs.		14

* Staging of controls and last treated sample to be decompressed for developmental rate studies.

$^{\Delta}$ For relative times of controls follow sub-column for last bottle, and disregard times for compression and decompression.

loaded. The contents of the bottles containing embryos plus medium still at a temperature near that of the water bath are emptied into a finger bowl containing about 50 ml. of fresh 10% Ringer's solution at room temperature. From this finger bowl, when they are at the mid- or late blastula stage, 100 (or, in a few experiments, 50) apparently normal eggs are selected. If, after decompression, less than 80% of the embryos appear normal in either the control or treated samples, no embryos are selected and the run is discontinued. In the selection process, the blastulae are transferred to a second finger bowl containing fresh 10% Ringer's solution. After staging of the embryos, all the finger bowls are placed at 18.0° C.

This results in a set of finger bowls, each of which corresponds to one of the pressure bottles. The 100 eggs in each appear normal and are in a uniform stage

of development earlier than gastrulation. These embryos are left to develop at 18.0° C. until neural fold formation of the controls.

Because of the selection process, pre-gastrular abnormalities are eliminated from the populations to be counted at the end of each run. Actually, oxygen treatment and shaking, either singly or in combination, are found by comparison with control groups of eggs to have little if any effect on development prior to gastrulation. The great majority of unhealthy eggs selected out are unfertilized. The selection process is completed before gastrulation.

Selection (as well as each of the other steps) is the same for the controls as for the treated eggs. The former are removed from the Warburg bath with the last of the series to be decompressed. Thus each member of these pairs of oxygen-treated and untreated embryos will have had the same history of temperature environments at the time of neurulation when abnormalities are counted. Collation of "stagings" on these sets from different runs constitutes the developmental rate studies.

3) When the control eggs are neurulas, cytolysis has not yet occurred in the abnormal embryos. At this time all the finger bowls are transferred to room temperature and the numbers of normal and neurulating embryos in each finger bowl are counted. If less than 95% of the controls are normal, the run is disregarded. In the abnormal class, evidence of developmental arrest or abnormality before stage 9 or 9 + is never found, and since only rarely does an abnormal neurula occur which does not show a gastrular aberration, that is, unincorporated yolk, the results are expressed as numbers or per cents of normal gastrulae.

Results

1. Identification of the Effective Agent

When *Rana pipiens* embryos are treated with oxygen during early cleavage they stop developing normally at the late blastula stage, well after decompression. The columns for oxygen in Table II show typical results with various dosage conditions.

Since effective oxygen treatment involves the use of pressure, the question arose as to the role that is played by this factor. Experiments were run using nitrogen instead of oxygen. They were otherwise identical in procedure and equal or greater in dosage than the oxygen runs which invariably produce embryos which fail to gastrulate normally. In none of the nitrogen experiments did more than 3% of the embryos block at gastrulation. Table II, which includes results with oxygen for comparison, shows no mechanical effect of pressure on development.

The ineffectiveness of pressure "alone" was confirmed by another type of experiment in which one bottle of a pair was shaken and the other was not. Except for this difference, the embryos in the two bottles had the same oxygen treatment. Several pairs of bottles were used, each pair for a different duration of treatment. Table III shows that even though pressure (and here the gas was oxygen) was the same in the stationary bottles as in the shaken ones, only in the former was gastrulation normal.

It might appear from consideration of Table III that shaking is the effective factor that we seek. But control embryos in air at atmospheric pressure are routinely shaken and show better than 95% normal development, for otherwise an

TABLE II

Per cent normal gastrulae with comparable treatments of oxygen and nitrogen. Air at 1 atmosphere. Treatment at 8.0° C.; 100 eggs per sample except at 45 p.s.i. of oxygen with 23 hours where 50 eggs used. Nitrogen samples from same cross. Oxygen samples from different crosses. All samples shaken at 30 c.p.m.

P. S. I. added to air	45				30		
Gas	O_2	N_2	O_2	N_2	O_2	N_2	N_2
Hours of treatment	14	14	23	24	23	24	38
Per cent normal gastrulae	25	97	0	99	5	99	99

experiment is disregarded. Gastrulation is normal even when shaking is combined with nitrogen pressure (see Table II).

Since shaking and pressure treatment singly or together are ineffective, and yet the two together with oxygen produce gastrular blockage, it becomes apparent that oxygen at the given pressures is the effective agent. The reason why the given pressures are required may be explained by Dalton's Law which states that in a two-phase system the solubility of a given gas in the liquid phase is directly proportional to its partial pressure in the gas phase above the liquid. Raising the oxygen pressure to the given hyperatmospheric levels, then, is one way to increase the solubility of oxygen in the medium so that at equilibrium the oxygen concentration is at a toxic level. Shaking the system speeds saturation of the medium after oxygen pressure is built up over it.

If, after oxygen pressure is applied, shaking serves merely as an aid in bringing the toxic agent, oxygen, through the 10% Ringer's solution to the embryos, then embryos which are not shaken should also be poisoned when treated for an additional period to allow for the slow reaching of equilibrium between the gas and liquid phases. To illustrate this point two series were run, one with shaking and the other without it. In each series several durations of oxygen treatment were used, a different sample of 50 embryos for each dosage. The two curves of Figure 3 show that to produce a given number of abnormal gastrulae it took roughly 5 hours more under oxygen pressure without shaking than it did with shaking. Two control series were also run at ambient air pressure, one series with shaking, and the other without shaking. In either series, at least 48 of the 50 embryos in each sample developed normally.

Before concluding that gastrular blockage is caused by excess oxygen in the liquid environment of the embryos, two miscellaneous possibilities must be eliminated. These are 1) that the embryos suffer from shock resulting from short compression and decompression periods and 2) that they are overcrowded (see Barth, 1946). As for the first consideration, reference to Table III, Figure 3, and the data in the next section (Dosage) reveals the many short doses of oxygen treatment, including those with shaking, which did not produce abnormal gastrulae. In

TABLE III

Per cent normal gastrulae with oxygen treatment with and without shaking. Treatment at 18.0° C. with 45 p.s.i. of oxygen added to air at 1 atmosphere; 100 eggs per sample. All samples from same cross. Shaking at 30 c.p.m.

Hours of oxygen treatment	10		13		16	
Shaking	−	+	−	+	−	+
Per cent normal gastrulae	92	0	93	0	95	0

all these cases, just as with the longer, harmful treatments, compression and decompression were gradual over 20 and 60 minutes, respectively. In addition, the embryos were treated in the same manner with nitrogen (*cf.* Table II) and development was normal. The possibility of overcrowding is precluded by these same data for, again, normal gastrulation resulted from conditions which were just as crowded as those which produced gastrular blockage. Furthermore, even the non-shaken, non-pressure-treated embryos showed at least 95% normal gastrulae as, of course, the shaken controls did. Actually, as far as oxygen concentration in the medium was concerned, the oxygen-treated embryos were "undercrowded." Thus it appears that neither duration of pressure change nor population density affected the embryos deleteriously.

It appears, then, that gastrular blockage is effected by a pressure-produced high oxygen concentration in the liquid environment of the embryos. Indeed, as

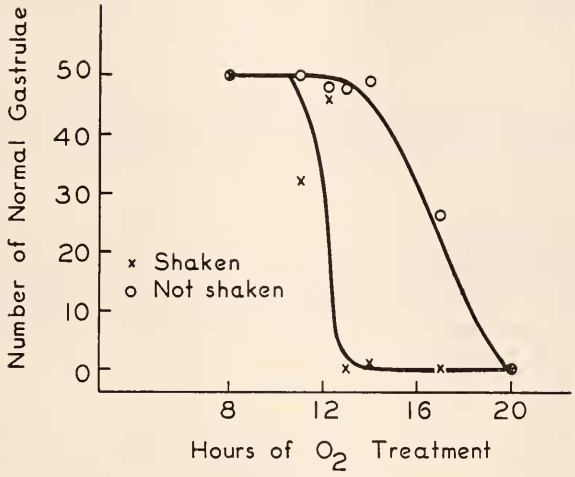

FIGURE 3. Effect of duration of oxygen treatment on gastrulation with and without shaking. Treatment at room temperature with 45 p.s.i. of oxygen added to air at 1 atmosphere; 50 eggs per sample. A different cross (no common parentage) used for each time dosage. Shaken and non-shaken samples at a given time dosage are from same cross. Shaking at 36 c.p.m.

will be shown below, the percentage of normal gastrulae varies inversely with the partial pressure of oxygen at a given duration of treatment (see Figure 8). If pressure does have some effect other than via Dalton's Law, certainly it is not through an increase in the force per unit area in the mechanical sense.

2. Dosage

Regardless of whether duration of oxygen treatment or pressure was varied, in each series or run, pressure was applied to all the samples simultaneously and before the first cleavage. Each dosage datum obtained represented a different sample of embryos since by the time the per cent normal gastrulae of a sample was determined, the normal embryos were too advanced to be blocked at the late

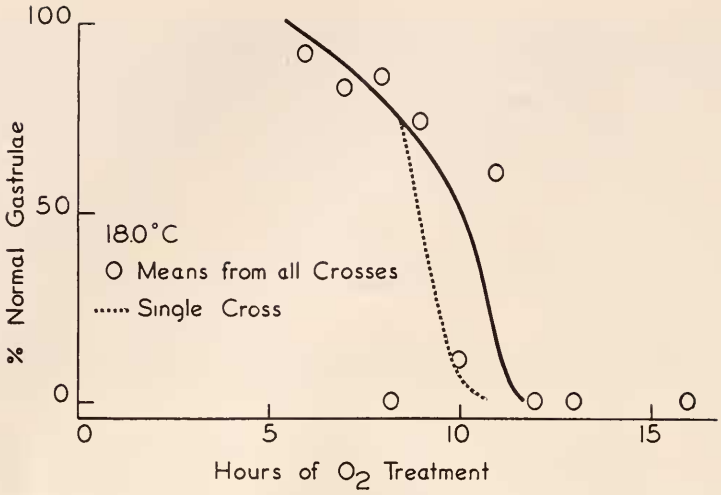

FIGURE 4. Effect of duration of oxygen treatment on gastrulation. The points represent the means from Table IV. Treatment at 18.0° C. with 45 p.s.i. of oxygen added to air at 1 atmosphere. Shaking at 30 c.p.m. Single cross from Figure 5.

blastula stage; thus a second dose on the same sample was precluded, and correspondingly, a second datum.

With few exceptions, the size of each sample was 100 embryos. Increasing a given sample to 200 made a difference of no more than 5% normal gastrulae. Occasional recounts of the same sample agreed within 2%. All the data in this section are from shaken samples.

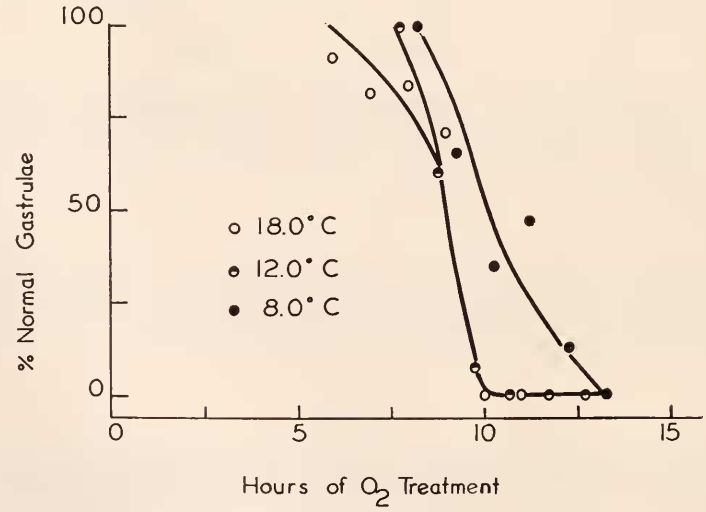

FIGURE 5. Effect of duration of oxygen treatment at various temperatures on gastrulation. Pressure is 45 p.s.i. of oxygen added to air at 1 atmosphere; 100 eggs per sample. A different cross (no common parentage) used for each temperature. Shaking at 30 c.p.m.

Curves of per cent normal gastrulae versus degrees of dosage of either kind in which all the embryos had the same parents were the most valuable, for genetic variability was thus avoided. Where, alternatively, the arithmetic averages of sets of repeat experiments were used, information was provided concerning any *R. pipiens* embryo of any parents, but the contours of the curve were softened and sharp breaks were obscured (see Figure 4).

TABLE IV

Per cent normal gastrulae with increasing durations of oxygen treatment and different parental backgrounds. Treatment at 18.0° C. with 45 p.s.i. of oxygen added to air at 1 atmosphere. Shaking at 30 c.p.m.

Exptl. run no.	Crosses	Hours of oxygen treatment									
		6	7	8	8¼	9	10	11	12	13	16
1	♀ 1 ♂ 1						0			0	0
2	♀ 2 ♂ 2		83			43					
3	♀ 3 ♂ 3	92	83	86		71	0	0			
4	♀ 4 ♂ 4 ♀ 5 ♂ 4 ♀ 6 ♂ 4					95 90 0		71 81 0			
5	♀ 7 ♂ 5 ♀ 8 ♂ 5 ♀ 9 ♂ 5 ♀ 10 ♂ 5				0		0 0 78 10		0		
6	♀ 7 ♂ 6 ♀ 8 ♂ 6				0 0		0 0		0		
7	♀ 11 ♂ 7 ♀ 11 ♂ 8 ♀ 11 ♂ 9					98 96 99		79 97 91			
Means		92	83	86	0	74	11	60	0	0	0
No. of samples		1	2	1	3	8	8	7	2	1	1

34 samples: 15 crosses: 11 ♀ ♀ (13 clutches; ♀7, ♀8 stripped twice)
100 embryos/sample 9 ♂ ♂
7 ♀ ♂ (No common parentage)

a. Duration of treatment (time dosage and per cent abnormality)

With increasing time dosage the per cent normal gastrulae dropped sharply. In Figure 4, a mean curve is plotted showing all the data at 18.0° C., with 45 p.s.i. of oxygen added to air at 1 atmosphere; 3400 embryos and 15 crosses are represented (see also Table IV). A curve of samples drawn from one of the 15 crosses (♀3 ♂3 in Table IV, 18.0° C. curve in Figure 5) is also presented. In either case, as soon as the embryos had been treated long enough to affect a few, it took

a relatively short additional dose ("effective time dosage") to affect them all. For the curve of the means this was 6 hours.

The steepness of the slopes in Figure 4 is confirmed by Figure 5. The latter shows curves from three sets of samples, each set from a different cross with no common parents. Each set was run at a different temperature but with the same pressure dosage. It is seen that from 100% to 0% normal gastrulae took 5 hours at 8.0° C., 3 hours at 12.0° C., and 4 hours at 18.0° C. (except that in the 18.0° C. set the highest figure is only 92%). As a matter of fact these figures are maximal. If they are in error due to the points being taken at intervals of no less than an hour, correction would only shorten the effective time dosage.

Additional evidence of the shortness of the effective time dosage is provided by Figure 6 which shows the data from all crosses run at 8.0° and at 18.0° C. At each temperature the pressure dosage was the same. Each cluster of points is concentrated along the time axis.

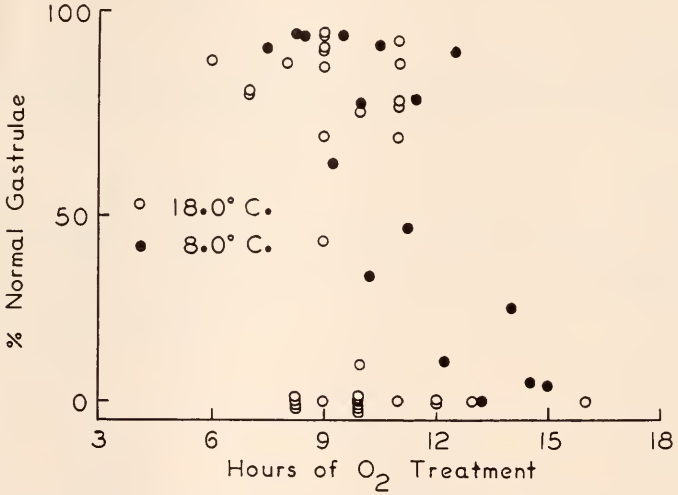

FIGURE 6. Effect of temperature on oxygen poisoning. Pressure is 45 p.s.i. of oxygen added to air at 1 atmosphere; 100 eggs per sample. Data for 18.0° C. from 15 crosses; for 8.0° C. from 5 crosses. Shaking at 30 c.p.m.

The other interesting aspect of these curves (Figs. 4, 5, 6) is the "lag dosage" before the oxygen is effective (see also Table IV). Its length of about 8 hours stands in contrast to the shortness of the effective time dosage. (Since all these data are from shaken samples, this lag is independent of that due to slow diffusion which is shown in the curve of Figure 3 for non-shaken eggs.)

b. The effects of temperature on time dosage

Oxygen solubility varies inversely with temperature. The increment in concentration of this gas is especially large from 18.0° to 8.0° C. in a saline solution (Umbreit, Burris and Stauffer, 1949, p. 5). Thus lowering the temperature has the same effect as increasing the pressure (*cf.* Dalton's Law) and this, it will be

seen in a later section, decreases the duration of treatment necessary for gastrular blockage (see Figure 8).

Also lowering the time dosage is a second effect of a drop in temperature. Sensitivity to oxygen decreases with developmental age (Nelsen, 1949). Since lowered temperature also slows development, any given duration of treatment is more effective at a lower temperature than at a higher one, for that part of the time at 18.0° C. spent on later stages is expended at 8.0° C. on the earlier, more sensitive stages. Consequently, less hours of treatment are required to produce gastrular blockage at 8.0° than at 18.0° C.

In two ways, then, temperature decrease enhances the effectiveness of oxygen treatment and tends to shift to the left a curve of per cent normal gastrulae versus hours of treatment (see Figure 7). On the other hand, unless they are very atypical, the actual chemical reactions resulting from oxygen treatment are slowed by a temperature decrease (Getman and Daniels, 1943, p. 363). This third effect tends to cancel out the other two. Thus any separation along the time axis of curves at different temperatures is a net effect.

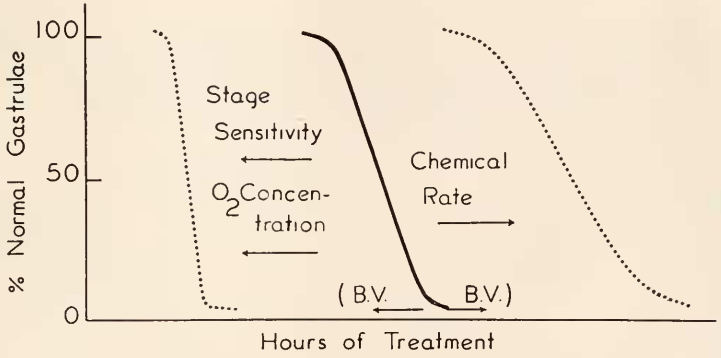

FIGURE 7. Postulated compensatory effects of decreased temperature on oxygen poisoning. B. V. stands for "Biological Variability." See text for explanation.

As far as lag dosage is concerned, neither Figure 5 nor Figure 6 shows any net effect of temperature. The former shows the results of oxygen treatment at three temperatures over a 10.0° C. range. The slight separation of the three curves is well within the variability inherent in the biological material (see Table IV) and therefore cannot be considered significant. Moreover, in Figure 6 error due to this variability is reduced through the use of samples from many crosses and here the cluster of points for 18.0° C. and those for 8.0° C. show the same lag dosage.

In Figures 5 and 6, although the lag dosage remains unaffected, the effective time dosage is increased by a temperature drop. In the former figure the slope of the 8.0° C. curve is less than that of the curves of 12.0° and 18.0° C. Although error is introduced due to the large time intervals (1 hour) between points, this error, as well as that due to biological variability, is reduced in Figure 6. Here, confirming the data of Figure 5, the cluster for 8.0° C. is more spread out along the abscissa than is the one for 18.0° C. In addition, with a fast drop in percentage

as compared to a slow drop, there is smaller probability at a random time dosage of a point falling midway between 100% and 0% normal gastrulae. The points for 18.0° C. actually do aggregate at the ends of the percentage range while those for 8.0° C. are more evenly spread along the ordinate.

Thus in the range from 8.0° to 18.0° C. the several effects of temperature are fully compensatory for lag dosage, while for the effective time dosage the chemical rate effect is greater than the combination of the opposite two (see Figure 7) and a net positive temperature coefficient for oxygen poisoning is demonstrated.

c. The effect of time dosage on type of abnormality

Even though the embryos from different crosses varied greatly in their oxygen sensitivity (see Table IV and Figure 3), a rather striking uniformity of response to treatment was demonstrated among siblings. This was especially well shown when, after tabulating the per cent of normal gastrulae (class 1), the abnormal embryos in each sample of a time dosage series were broken down according to

TABLE V

Per cent of gastrulae of each class in samples exposed to increasing durations of oxygen treatment at 12.0° C. Pressure is 45 p.s.i. of oxygen added to air at 1 atmosphere; 100 eggs per sample. All samples from same cross and shaken at 30 c.p.m.

Classes of gastrulae	Hours of oxygen treatment					
	$7\frac{3}{4}$	$8\frac{3}{4}$	$9\frac{3}{4}$	$10\frac{3}{4}$	$11\frac{3}{4}$	$12\frac{3}{4}$
1. Normal	99	60	7	0	0	0
2. Incompletely blocked	1	40	93	63	42	1
3. Completely blocked	0	0	0	37	58	99

type of gastrular blockage as follows: class 2: incompletely blocked (abnormal gastrulation) and class 3: completely blocked (stage 9 or 9 +, no dorsal lip). As usual, each sample was drawn from the progeny of the same cross and represented a different time dosage; except for the latter, the conditions of treatment were kept constant.

As is shown in Table V, with increasing time dosage each of the three classes is progressively filled, leaving always at least one null class. After class 2 reaches nearly 100% it decreases as class 3 increases. At $9\frac{3}{4}$ hours there are two null classes (1 and 3) for practically all the embryos have been treated long enough to prevent normal gastrulation, but none have yet been affected so badly as to prevent it completely. The change from $8\frac{3}{4}$ to $9\frac{3}{4}$ hours of treatment is entirely from class 1 to class 2. Those embryos already in class 2 at $8\frac{3}{4}$ hours do not enter class 3 with the additional hour of treatment. They "wait" for the embryos still normal with $8\frac{3}{4}$ hours of treatment to "catch up" and become incompletely blocked at $9\frac{3}{4}$ hours, that is, until class 2 is full before becoming completely blocked at $10\frac{3}{4}$ hours.

These data reveal two discrete thresholds of time dosage, an earlier one for incomplete blockage of gastrulation, and a later one for complete blockage. In the data of Table V, these occur, respectively, between $7\frac{3}{4}$ and $8\frac{3}{4}$, and between $9\frac{3}{4}$ and $10\frac{3}{4}$ hours of oxygen treatment.

Occasionally in runs at 8.0° C. with 45 p.s.i. of oxygen added to 1 atmosphere of air the embryos are distributed among all three classes at one time dosage. Otherwise, however, the pattern of progression from 100% normal to 100% incompletely blocked to 100% completely blocked gastrulae recurs in time dosage series run at 18.0° or 12.0° C. with 45 p.s.i. of oxygen added to air at ambient pressure or at 8.0° C. with 30 or 15 p.s.i. added to air. In addition, the same kind of results are obtained when the abnormal embryos are further subdivided into four classes.

d. Pressure dosage

In these studies, 45, 30, and 15 p.s.i. of oxygen were used; each was added to air at 1 atmosphere. Since the partial pressure of oxygen in the latter is about

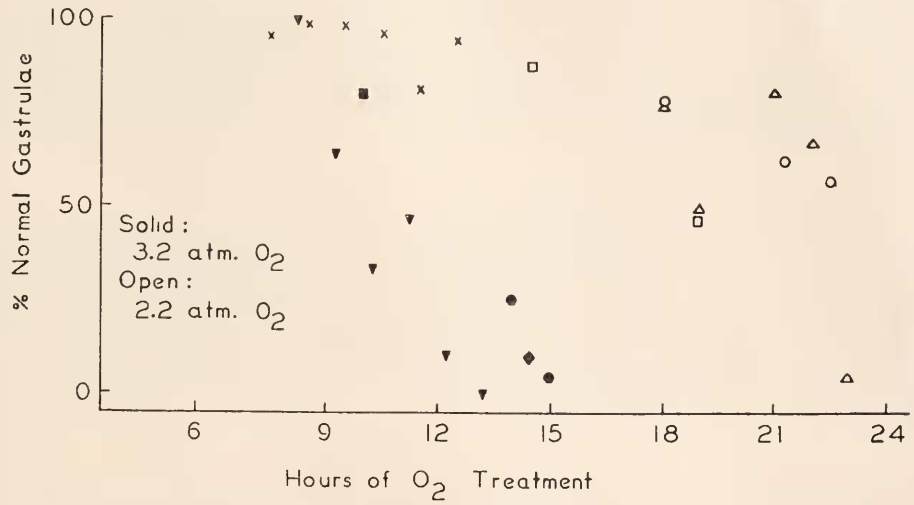

FIGURE 8. Gastrular blockage as a function of partial pressure of oxygen for various durations of treatment. Treatment at 8.0° C.; 100 eggs per sample. Samples from different crosses (no common parentage) represented by symbols of different shape. Shaking at 30 c.p.m.

0.2 atmosphere, the addition of pure oxygen in the several cases resulted in partial pressures of approximately 3.2, 2.2, and 1.2 atmospheres.

Various time dosages were used with each pressure dosage except that of 1.2 atmospheres of oxygen. Each sample of embryos went through one period of treatment. The pressure was not changed during this period. Either one or several pressures of oxygen were used in a single run. All the samples in a run were from the same cross and a different cross (no common parentage) was used for each run.

Earlier, a partial pressure of 3.2 atmospheres of oxygen was found to be effective at 18.0° C. and at 12.0° C. These studies, however, were to include lower pressures and correspondingly weaker oxygen tensions in the 10% Ringer's solution. (Dalton's Law holds for oxygen to about 99% of the theoretical values in the pressure range of this work (Moore, 1950, p. 121).) In compensation, therefore, a temperature of 8.0° C. was used to ensure effectiveness of the treatment. The temperature reduction was expected (see Figure 7) to act in these ways: 1) to increase the oxygen concentration in the 10% Ringer's solution, and 2) because of decreased developmental rate, a) to concentrate the treatment on the earlier, more sensitive stages, and b) to increase the number of hours of treatment possible before gastrulation. These effects were considered of more importance than the antagonistic one of decreased chemical rate.

Figure 8 presents all the data with 3.2 and 2.2 atmospheres of oxygen plotted as per cent normal gastrulae against duration of treatment. The points fall into two separate clusters corresponding to the oxygen dosages used. An effect was also obtained with 1.2 atmospheres of oxygen. After 88 hours of treatment the embryos were in the mid-blastula stage and appeared normal. After selection at stage 9, however, none gastrulated normally.

These data show that for a given time dosage, the percentage of embryos poisoned by oxygen varies directly with the partial pressure of that gas. This, of course, is consistent with the evidence presented in a previous section that the role of pressure in effective oxygen treatment lies in its increasing the oxygen concentration in the egg medium.

c. Pressure-time-dosage relationships

Increased duration of treatment compensates for reduced partial pressure of oxygen. The most extreme demonstration was the experiment in which a dosage of 1.2 atmospheres resulted in gastrular blockage with 88 hours of treatment. Thus, for a given effectiveness of oxygen poisoning in terms of per cent normal gastrulae, pressure dosage varies inversely with time dosage. This may be seen by extending a horizontal line through the 2 clusters of Figure 8 and comparing their time and pressure dosages at that level.

3. Rate of Development

In almost every experimental run, the stages of the embryos were determined at one or two developmental ages before gastrulation. Each run provided one or more sets of one untreated, or control, and one oxygen-treated sample, each set representing a different parental cross. The members of a given set were of equal sample sizes. At the times of comparative staging, the two samples in each set had the same history of temperature environments. The results of 35 stagings on 22 crosses are collated in Table VI.

In those cases in which retardation did occur, it was of the oxygen-treated eggs. With a few exceptions (cross no. 9, 10, 21), retardation did not occur in those 11 sets in which the treated samples went on to show some percentage of normal gastrulae. Even in the exceptional cases, the retardation appeared only in the later (B) staging. In 11 crosses providing 17 stagings, the oxygen treatment resulted in 0% normal gastrulae. In 7 of these 11 crosses, the treated sample

TABLE VI

Comparison of developmental stages of oxygen-treated and untreated embryos, the samples of a given cross having the same history of temperature environments at the time of staging. All samples in a given run fertilized at the same time. One treated and one untreated sample per cross. Staging shortly after decompression denoted by letter A; after selection by letter B (cf. Table I). About 300 eggs per sample at A staging; 100 eggs per sample at B staging except for cross no. 18 where 50 eggs were used and cross no. 21 where 192 treated and 200 untreated eggs were used. All samples shaken at 30 c.p.m.

Run no.	Cross no.	Oxygen treatment			Per cent normal gastrulae of treated eggs	Developmental stages of eggs	
		Temperature	Atm.	Hours		Treated	Untreated
1	1B	18.0° C.	3.2	16	0	9−	9
2	2B	"	"	9	43	8+	8+
3	3B	"	"	11	0	9−	9−
4	4B	"	"	"	71	9−	9−
	5B	"	"	"	81	9−	9−
	6B	"	"	"	0	9−	9−
5	7A	"	"	10	0	8+	8+
	7B	"	"	"	"	9	9+
	8A	"	"	10	0	8+	8+
	8B	"	"	"	"	9	9+
	9A	"	"	10	78	8+	8+
	9B	"	"	"	"	9	9+
	10A	"	"	10	10	8+	8+
	10B	"	"	"	"	9	9+
6	11A	"	"	12	0	8	8+
	11B	"	"	"	"	9	9+
7	12A	"	"	11	79	8+	8+
	12B	"	"	"	"	9+	9+
	13A	"	"	11	97	8+	8+
	13B	"	"	"	"	9+	9+
	14A	"	"	11	91	8+	8+
	14B	"	"	"	"	9+	9+
8	15B	12.0° C.	"	14	0	8	8
9	16B	"	"	$12\frac{3}{4}$	0	8+	9−
10	17B	8.0° C.	"	$12\frac{1}{2}$	94	8+	8+
11	18A	"	"	$23\frac{1}{2}$	0	5	6
	18B	"	"	"	"	7+	8

TABLE VI (*Continued*)

Run no.	Cross no.	Oxygen treatment			Per cent normal gastrulae of treated eggs	Developmental stages of eggs	
		Temperature	Atm.	Hours		Treated	Untreated
12	19A	8.0° C.	3.2	13¼	0	4	4
	19B	"	"	"	"	9−	9−
13	20A	"	2.2	23	5	7	7
	20B	"	"	"	"	8	8
14	21A	"	"	19	47	6	6
	21B	"	"	"	"	9−	9
15	22A	"	1.2	88	0	9−	9
	22B	"	"	"	"	9−	9+

was retarded. Of the 17 stagings, in 10 cases the controls were ahead of the treated animals.

Where retardation did appear (in 13 of the 35 stagings), it was slight. Except for one staging (no. 18A), the delayed samples were less than one Shumway (1940) stage behind the controls.

In 10 of the 22 crosses development of the oxygen-treated samples was delayed as compared to that of the controls. However, only in cross no. 18 was retardation shown before blastulation. In all cases where the beginning of the lag could be well localized (cross no. 7, 8, 9, 10), it first appeared in the blastula stage.

The substaging (9 −, 9, 9 +) technique was neither very accurate nor precise from run to run. On the other hand, comparing the stages of the two samples at any given time was quite reliable. In other words, in Table VI, comparison of stages in horizontal rows is more dependable than in the vertical ones. Therefore, it is harder to accurately compare sets of samples from many crosses in order to tell when retardation first appears, than it is to tell in how many of all the crosses retardation does occur, and how slight it is. A few experiments without shaking were performed. Here again, in those cases when it occurred, retardation of the oxygen-treated eggs was slight and during blastulation. In the runs using nitrogen (see Table II) instead of oxygen, the gas-treated samples showed no lag in development.

Comparative staging was not as accurately performed at neurulation when the percentages of normal gastrulae were determined as at the pre-gastrular stages recorded in Table VI. Nevertheless, at that time no significant differences in developmental stage between the control, and treated but unharmed eggs were noticed.

It is seen, therefore, that in many but not all cases, oxygen-treated embryos are retarded as compared to untreated controls. Generally, however, the decrease in developmental rate is slight and begins during the late blastula stage.

DISCUSSION

Amphibian embryos subjected at fertilization or during cleavage to any one of a variety of treatments will not gastrulate. This process seems to be a critical

one in early development and if not all, certainly larger percentages of experimentally treated eggs die or first appear abnormal at this stage than at any other. Conditions bringing this about include certain hybridizations (Moore, 1941); Brachet, 1944), CN^- treatment (Spiegelman and Moog, 1945), parthogenesis (Parmenter, 1933), and uterine over-ripening (Briggs, 1941). The developmental block produced by oxygen pressure in *R. pipiens* embryos is another which is manifested at or near gastrulation.

The normal development demonstrated in the experiments using nitrogen pressure, some of those using oxygen pressure without shaking, and those of Nelsen (1948) using air pressure, shows that the inhibition of gastrulation through the use of oxygen pressure is not a mechanical effect; oxygen poisoning is not the result of the exertion of a high force per unit area. This is not surprising, for living systems are relatively unaffected by non-localized pressures applied and released gradually (Heilbrunn, 1952, pp. 503–509). With few exceptions, the lowest such pressures having a biological effect are about 100 times those used in the present studies.

At a given temperature the oxygen tension of the embryos' culture medium is directly proportional to the partial pressure of the gas (Dalton's Law) when equilibrium is established, and the results of these experiments can be explained by assuming that the gastrular abnormalities studied are, in turn, functions of oxygen tension. This assumption is confirmed by the experiments using oxygen with and without shaking. Equilibrium between gas and liquid phases is more rapidly established with shaking and the oxygen tension quickly reaches its saturation level in the 10% Ringer's solution. Thus for threshold durations of oxygen treatment, gastrular blockage occurs only in the shaken embryos. As would be expected, non-shaken embryos will be affected only if they are treated with oxygen for longer periods of time. Furthermore, with higher oxygen pressure, the percentage of normal gastrulae falls (see Figure 8). Additional confirmation is provided by experiments of Nelsen (1949) which have been repeated by the present writer. These employed a vertically suspended string-like mass of eggs exposed to oxygen pressure without shaking. Those at the top of the string near the surface of the medium, and therefore in contact with a saturated solution of oxygen, stopped developing at gastrulation. The lower the position of the embryos and, correspondingly, the lower the oxygen tension, the more normal were their fates at gastrulation.

The dosage studies reveal a threshold whose significance is not clear. For, it takes a relatively long duration of treatment (about 8 hours) to produce any abnormal gastrulae; yet after this dosage is completed, treatment of only about 4 more hours results in no normal gastrulae.

Frequently the dosage necessary to produce 100% completely blocked gastrulae is greatly exceeded. Yet developmental arrest never occurs earlier than at the late blastula stage. This suggests that until the very end of the pre-gastrular period, as opposed to the post-gastrular stages, normal development of the embryo is not dependent upon systems which are sensitive to high oxygen tensions.

The developmental rate studies confirm this view. Almost without exception, oxygen-treated embryos develop at the same rate as do untreated ones—until late blastulation. At that time, some, but not all, embryos are slightly retarded. This is understandable if some system sensitive to high oxygen tension, although nec-

essary for normal development after gastrulation, may be dispensed with before this process if, indeed, it operates during the pre-gastrular stages at all.

It should be pointed out that results of this sort are not obtained with all agents causing developmental arrest at gastrulation. Although certain hybrids, as well as oxygen-poisoned embryos, do stop developing abruptly (Moore, 1941), CN^--treated animals are invariably retarded by several stages beginning in early cleavage (Spiegelman and Moog, 1945). Thus in the case of CN^- it cannot be said that what is being affected at the chemical level is correlated in the normal embryo specifically with the events beginning at gastrulation.

The interpretation for the CN^- experiments may be that before gastrulation the poison inhibits a cytochrome oxidase-limiting system which controls developmental rate to an extent such that the latter is merely decreased. At gastrulation, however, the level of inhibition relative to the heightened energy demands (see Barth and Barth, 1954) is such that development ceases entirely. This is a concept of a *quantitative* change at gastrulation.

With oxygen poisoning the interpretation is that a *qualitative* change occurs at or just prior to gastrulation such that a chemical system comes into play whose operation is necessary for development to proceed, but which is not needed for even unretarded pre-gastrular development. What is inhibited during early cleavage is either this system sensitive to high oxygen tension or the conditions necessary for the system's establishment.

These studies were designed as the preliminary steps toward analyses at the cellular and chemical levels. As to the former, the possibility must be entertained that in oxygen-poisoned embryos, gastrular blockage is mediated through chromosomal aberrations. For, increased oxygen tensions enhance x-irradiation effects (Giles and Riley, 1950), and Conger and Fairchild (1952) showed that the chromosome breakage produced by oxygen in *Tradescantia* microspores was identical to that caused by x-rays. Thus it has been suggested (Gerschman, Gilbert, Nye, Dwyer and Fenn, 1954) that high oxygen tensions act similarly to x-rays.

As for the chemical considerations, Brachet, who has long emphasized the role of —SH in development (1950, pp. 170–184), has suggested that the —SH enzymes are inactivated in the oxygen-poisoned embryos (1949). This seems quite probable for Haugaard (1946), using adult mammalian tissue slices and homogenates, demonstrated a close correlation between susceptibility to inactivation by high oxygen pressure and the presence of essential —SH groups in some 20 oxidative and non-oxidative enzymes. Dickens, also in 1946, presented similar results. Non-protein —SH groups are also affected by oxygen, the rate of oxidation being proportional to the oxygen pressure (Barron, 1955). It is generally believed that the inactivation operates through an irreversible oxidation of —SH to —S—S—.

With this body of work as a guide, a metabolic analysis has been started (Malamed, 1954). It was found that oxygen-treated embryos had the same oxygen uptake rate as controls, from shortly after decompression until the late blastula stage. After this stage the controls continued to rise in respiratory rate. At this point, however, corresponding to the time when all the treated embryos were completely blocked, their rate of oxygen consumption levelled off. It then stayed constant until cytolysis set in, about the time the controls developed tailbuds. These results, the same as obtained with a frog hybrid by Barth (1946), indicate that what the oxygen-sensitive system is needed for is the (aerobic) production of

energy, which is in turn presumably necessary for the cell movements or, more properly, the mechanical work which constitutes gastrulation.

I wish to express my appreciation for the encouragement and guidance, at both the theoretical and technical levels, of Prof. L. G. Barth. To Prof. O. E. Nelsen I am indebted for the first stimulation of an interest in embryos and oxygen poisoning. Special thanks are due to Prof. J. R. Gregg, Drs. R. McMaster, J. Reiner, and A. Kostellow for their criticisms and suggestions. Mr. A. Pfeiffer was most helpful in the design and construction of the apparatus.

Summary

1. The effect of oxygen poisoning on gastrulation in *Rana pipiens* eggs has been studied using an apparatus consisting of 6 pressure systems continuous with each other or not, in various combinations. The apparatus permitted the embryos to be kept at constant temperature. Shaking and non-shaking samples could be run simultaneously. Oxygen treatment started before the first cleavage and ended during the early cleavage stages.

2. In the mechanical sense, pressure has no effect on gastrulation, for gastrulation is normal in experiments using nitrogen and in others using oxygen without shaking.

3. The role of pressure is via an increase in the oxygen tension of the eggs' medium, according to Dalton's Law. That gastrular blockage is a function of oxygen tension is shown by comparing results with and without shaking for various durations of treatment and by the higher percentage of abnormal gastrulae with higher partial pressure of oxygen.

4. With shaking and 45 p.s.i. of oxygen added to air at 1 atmosphere, durations of treatment of less than 8 hours are without effect on gastrulation. At this threshold, additional treatment of about 4 hours results in no normal gastrulae.

5. Temperature has little if any (net) effect on oxygen poisoning. This is explained on the basis of several temperature effects which are largely compensatory.

6. With 2.2 atmospheres partial pressure of oxygen a longer duration of treatment is required to affect gastrulation than with 3.2 atmospheres. An effect has been obtained using 1.2 atmospheres.

7. Comparison with controls shows that after oxygen treatment the embryos are not always retarded before gastrulation. When there is a developmental delay, it is slight and does not begin before the late blastula stage.

8. These results are interpreted as follows: at gastrulation a qualitative change occurs such that a new chemical system on which development is dependent comes into play. During early cleavage high oxygen concentrations inhibit either this system or conditions necessary for its establishment.

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